cytoplasmic DNA synthesis
in amoeba proteus

III. Further Studies on the Nature
of the DNA–Containing Elements

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ABSTRACT

The application of electron microscope autoradiography to Amoeba proteus cells labeled with tritiated thymidine has permitted the identification of morphologically distinct particles in the cytoplasm as the sites of incorporated DNA precursor. The particles correspond to those previously described from light microscope studies, with respect to both H³Tdr incorporation and distribution in centrifugally stratified amoebae. Ingested bacteria differ from the particles, in morphology as well as in the absence of associated label. Attempts to introduce a normal particle labeling pattern by incubating amoebae with labeled sediment derived from used amoeba medium failed. The resultant conclusion, that the particles are maintained in the amoeba by self-duplication, is supported by the presence of particles in configurations suggestive of division.

We have previously described particulate elements in the cytoplasm of Amoeba proteus which contain cytochemically demonstrable DNA, and have presented light microscope autoradiographic evidence associating these particles with the incorporation of tritiated thymidine (H³Tdr) (1). The relationship between the number of particles and the amoeba's size and stage in the division cycle and the frequent occurrence of paired particles were interpreted as indicative of some degree of integration between the particles and the amoeba and as pointing against their direct and continuous origin from outside the cell (2). The increased resolving power afforded by electron microscope autoradiography has now enabled us to confirm the presence of incorporated H³Tdr in morphological entities in the cytoplasm of A. proteus which are readily distinguishable from normal bacterial structures found there. The earlier conclusion that the light microscopic labeling pattern obtained is not attributable to the ingestion of labeled micro-organisms has been strengthened by negative results obtained when amoebae were incubated with the highly radioactive, sedimentable material derived from medium in which amoebae had been grown in the presence of H³Tdr.

MATERIALS AND METHODS

Amoebae (Amoeba proteus) used for electron microscopy were grown as previously described (1). H³Tdr (New England Nuclear Corp., Boston, 6700 µc/µm) was administered to whole and anucleate cells at a concentration of 10 µc/ml over a period of 26 hours. Labeled cells were washed free of external isotope and centrifuged for 20 minutes at 10,000 g (1). The cells were then fixed either in 6.5 per cent glutaraldehyde (phosphate-
buffered to pH 7.1) for 2 hours at 5°C and then postfixed in Kellenberger's OsO₄ (1 per cent) for 30 minutes, or directly in Kellenberger's OsO₄ for 30 minutes at 5°C. Following OsO₄ fixation, the amoebae were treated with uranyl acetate (3), dehydrated in ethanol, and imbedded singly in capsules in either methacrylate-divinyl benzene (4), Epon (5), or Maraglas Cardolite (6). Thin sections (approximately 80 μm) of oriented amoebae were cut on a Servall Porter-Blum microtome using a Servall diamond knife and mounted on copper grids covered with carbon-coated formvar films. Electron micrographs were taken with a Siemens Elmiskop IIb at original magnifications of × 7,200 and × 15,000 on Kodak lantern slide plates and developed in D-19. Ilford L-4 emulsion was applied to grids according to the method of Caro (7). The grids were exposed for 87 days over silica gel at 4°C, developed in Microdol X for 5 minutes, fixed in F.R. Rapid Fix for 1.5 minutes, and washed in 10 changes of distilled water over a period of 10 minutes.

The amoebae used for light microscope autoradiography were labeled as nucleate or anucleate half cells, either by incubation with H₃Tdr for 24 hours as above or for a similar period in a medium containing label only in association with a sedimentable fraction derived from the above incubation medium. This fraction was obtained as a pellet by centrifugation (32,500 g for 30 minutes) of the H₃Tdr medium in which amoebae had been incubated for 24 hours, after removal of the amoebae. The pellet was resuspended, washed, and centrifuged four times in amoeba medium containing unlabeled Tdr (5 × 10⁻⁶ M). Amoebae were then added to the pellet suspended in the last wash. Microscopic examination of the suspended pellet material revealed the presence of motile bacteria as well as of a heterogeneous mixture of particulate matter. The pellet could also be shown to contain appreciable radioactivity by counting.

Centrifugation, fixation, slide preparation for autoradiography, and DNase digestion were performed as previously outlined (1).

RESULTS

The major morphological characteristics of a centrifuged Amoeba proteus as seen with the electron microscope are sketched in Fig. 1. The diagram represents the stratification pattern in the average cell; it agrees—with respect to nuclei, crystals, mitochondria, and Golgi bodies—with the pattern described earlier by Daniels (8). In both whole and anucleate cells, the region in which light microscopy of centrifuged amoebae had shown high concentrations of DNA-containing particles (1) was found to contain large numbers of bodies not previously described from electron microscope observations (Figs. 2, 3, and 5). These “bodies” have a mean diameter of about 0.5 μ; they tend to appear in clusters and often show forms suggestive of division (Fig. 5). They are contained in vacuoles—usually singly—the limiting membranes of which appear to be derived from the amoeba’s ergastoplasm. The “bodies” are delimited by a double membrane within which it is possible to discern fibers having a diameter of about 70 A which can sometimes be resolved into two component fibers. Spherical particles which resemble ribosomes in their morphology and affinity for uranyl acetate are also present. These new structures are morphologically distinct from bacteria which are found in various stages of digestion within vacuoles in the whole centrifugal half of the amoebae (Fig. 4). (The morphology of these structures makes their identity with those described by Roth and Daniels questionable (9).)

The electron microscopic examination of autoradiographic preparations of amoebae stratified after incubation with H₃Tdr showed that 51.5 per cent of the silver grains was within 0.5 μ of centers of the aforementioned “bodies” (Figs. 6 and 7, b, 7, and 8); the other grains were not associable with any recognizable structural entity. One

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**FIGURE 1** Outline diagram of a longitudinal section of a centrifuged *Amoeba proteus* showing the average stratification pattern of the various cell components as seen in the electron microscope. The arrow indicates the direction of centrifugal force.

**FIGURE 2** Electron micrograph of a section made in the particle region (cf Fig. 1) of a centrifuged *Amoeba proteus*. A number of “bodies” (B) can be seen as well as ergastoplasm (er); dense bodies (d); Golgi bodies (g); and mitochondria (mit). (Kellenberger’s OsO₄; Maraglas-Cardolite) × 30,000.
hundred and two of 198 grains found over a total of 47 sections were associated with the new structures. The significance of this grain distribution becomes evident when one considers that the background grains were present in an area at least 12 times that occupied by the "bodies"; i.e., the average background grain density was 1/12 that of the "bodies." No labeled bacteria were seen.

Light microscopic examination of autoradiographic preparations of both nucleate and anucleate amoebae incubated with free H₃Tdr showed incorporation which agreed with previously reported patterns with respect to both DNase sensitivity and stratification (1). Anucleate amoebae incubated with the sedimentable fraction of labeled amoeba medium but without added H₃Tdr did not become labeled; nucleate amoebae so treated, on the other hand, became heavily labeled. This label, however, could not be stratified nor was it removable by DNase digestion.

DISCUSSION AND CONCLUSION

Light microscope studies of Amoeba proteus which had incorporated H₃Tdr into the cytoplasm provided evidence for cytochemically demonstrable DNA in small particulate elements. The spatial correlation between the incorporated radioactivity and concentrations of particles in centrifugally stratified amoebae led to the conclusion that the H₃Tdr was associated with the particles. Electron microscope autoradiography of nucleate and anucleate amoebae centrifuged after incubation with H₃Tdr has now established the presence of discrete, labeled bodies in the region of the stratified cell where the DNA-containing particles would be expected. The distribution of silver grains over the newly described "bodies" as compared with that over other cytoplasmic areas leads us to conclude that the "bodies" are the only significantly labeled entities in the amoeba's cytoplasm. There appears little doubt that the "bodies" seen in the electron microscope correspond to the DNA-containing particles described previously and that they are, in fact, the site of H₃Tdr-labeled DNA.

(It should be emphasized that this conclusion applies only to amoebae labeled under conditions in which all the incorporated H₃Tdr can be stratified and is removable by DNase digestion. Beyond its insensitivity to DNase, we have no data at present for establishing the nature of the non-stratifiable label. The resistance of this material to enzymatic digestion implies that it is either not DNA at all or DNA which is inaccessible to the enzyme.)

There are two lines of evidence which indicate that the DNA-containing particles duplicate within the amoeba cytoplasm. The previously reported maintenance of the particles from one cell generation to the next implies either internal duplication or continuous origin from outside the amoeba. The latter possibility seems unlikely in view of the demonstrated absence of significant acid-insoluble labeling of anucleate cells exposed to a medium rich in labeled sedimentable material. It should be kept in mind that such anucleates, when exposed to free H₃Tdr, incorporate this precursor readily into DNA-containing particles (10; see also Fig. 8). The fact that the extensive labeling found in nucleate cells incubated with labeled sedimentable material is insensitive to DNase and not readily stratifiable points in the same direction; however, the density of the ingested label in these cells limits the certainty with which the absence of particle labeling can be established. The second argument for internal duplication derives from frequent observations of particle configurations suggestive of division.

The origin and significance of these DNA-containing particles in the cytoplasm of Amoeba proteus are still matters of conjecture. The presence of the particles within vacuoles and what can be seen of their internal fine structure are suggestive of their origin as symbionts. Alternatively, it is conceivable that the particles were of nuclear origin at some time in the amoeba's evolution, particularly in view of recent findings of but a single band in a cesium chloride gradient analysis of DNA extracted from starved amoebae (Mandel,

![Figure 3](http://jcb.rupress.org/figure/3)

**Figure 3** Electron micrograph showing a group of "bodies." Fibers, about 70 Å in diameter, and ribosome-like particles can be discerned within the double limiting membrane (mb) of the "bodies." The arrows point to where the duality of the fibers is evident. The bodies are separated from the surrounding cytoplasm by a vacuole (v), delimited by a double membrane (me). Dense spheres (d) and a Golgi body (g) are also shown. (Kellenberger's OsO₄; Epon) X 60,000.
personal communication). Since, however, visual impressions of acridin orange-stained amoebae suggest that particle DNA is present in larger quantities than nuclear DNA, it may be necessary to establish that both nuclear and particle DNA's were, in fact, detected in the analysis before compositional identity of the two DNA's can be accepted as a basis for serious speculation.

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REFERENCES


Figure 4 A group of bacteria in various stages of digestion (cf “bodies,” Fig. 3). These bacteria were in a vacuole in the amoeba’s cytoplasm. (Kellenberger’s OsO4; Epon) × 60,000.

Figure 5 A division-like figure of a “body.” Fibers and ribosome-like particles are discernible within the limiting membranes (mb). The arrows point to where it is evident that the limiting membrane of the “body” (mb) and the limiting membrane of the vacuole surrounding the “body” (me) are double membranes. The ribosomes (r) on the vacuolar membranes suggest the possible ergastoplasmic nature of these membranes (cf ergastoplasm (er) in amoeba’s cytoplasm). (Glutaraldehyde, Kellenberger’s OsO4; methacrylate-divinyl benzene) × 60,000.
FIGURES 6 a and 6 b  Electron microscopic autoradiographs of adjacent sections of a group of "bodies" in a centrifuged amoeba. (Glutaraldehyde, Kellenberger's OsO₄; methacrylate-divinyl benzene) × 36,000.

FIGURE 7 Electron microscopic autoradiograph of "bodies" in a centrifuged amoeba. (Glutaraldehyde, Kellenberger's OsO₄; methacrylate-divinyl benzene) × 36,000.

FIGURE 8 Electron microscopic autoradiograph of a "body" in a centrifuged anucleate amoeba. (Glutaraldehyde, Kellenberger's OsO₄; methacrylate-divinyl benzene) × 36,000.